

ALKBH5-HOXA10 loop-mediated JAK2 m6A demethylation and platinum resistance in epithelial ovarian cancer

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Research

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Abstract

Background: Chemotherapy resistance remains a barrier in improving the prognosis of epithelial ovarian cancer (EOC), but its mechanism remains to be elucidated. ALKBH5 has been recently proven to be an RNA N6-methyladenosine (m6A) demethyltransferase associated with various cancers, but its role in cancer therapeutic resistance remains unclear. This study aimed to investigate the role of AlkB homolog 5 (ALKBH5) in platinum-resistant EOC.

Methods: Functional assays were performed both in vitro and in vivo. RNA sequencing (RNA-seq), m6A-modified RNA immunoprecipitation sequencing (MeRIP-seq), chromatin immunoprecipitation, RNA immunoprecipitation, and luciferase reporter and actinomycin-D assays were performed to investigate RNA/RNA interaction and m6A modification of the ALKBH5-HOXA10 loop.

Results: ALKBH5 was upregulated in platinum-resistant EOC and promoted cancer cell cisplatin resistance both in vivo and in vitro. Notably, HOXA10 was found to be the upstream transcription factor of ALKBH5 and formed a loop with ALKBH5, and its overexpression facilitated EOC cell chemoresistance both in vivo and in vitro. HOXA10 overexpression was found to facilitate EOC cell chemoresistance both in vivo and in vitro. Collective results of MeRIP-seq and RNA-seq showed that JAK2 is an m6A-modified gene targeted by ALKBH5. The JAK2/STAT3 signaling pathway was activated by overexpression of the ALKBH5-HOXA10 loop, and this resulted in EOC chemoresistance. Cell sensitivity to cisplatin was rescued by ALKBH5 and HOXA10 knockdown or inhibition of the JAK2/STAT3 signaling pathway in EOC cells overexpressing ALKBH5-HOXA10.

Conclusions: The ALKBH5-HOXA10 loop jointly activates the JAK2/STAT3 signaling pathway by mediating JAK2 m6A demethylation, promoting EOC resistance to platinum. Thus, inhibition of the expression of the ALKBH5-HOXA10 loop maybe a potential strategy to overcome platinum resistance in EOC.

1. Background

Epithelial ovarian cancer (EOC) is the most fatal gynecological malignancy worldwide [1]. Platinum-based regimens are currently the first-line chemotherapeutic modality for EOC. However, while it is initially effective, most EOC patients have a poor prognosis owing to the inherently high rate of recurrence and resistance to platinum agents [2]. Unfortunately, evidence supporting the appropriate approach against platinum chemoresistance in EOC is limited because of its complicated mechanism. Therefore, it is crucial to explore the underlying mechanism as it could help identify useful therapeutic targets to overcome resistance and improve prognosis.

Several mechanisms, including activation of oncogenes, mutation of antioncogene, and dysregulation of cancer-associated signaling pathway, are involved in chemoresistance [3–5]. N6-methyladenosine (m6A) modification as one of the most prevalent modifications in mRNAs influences mRNA transcription, stabilization, and translation [6]. The core molecules of the m6A methyltransferase complex, including

methyltransferase-like 3, methyltransferase-like14, and WT1-associated protein, mediate the methylated modification [7]. Meanwhile, m6A erasers, including fat mass and obesity-associated and AlkB homolog 5 (ALKBH5), act as demethylases to reverse the m6A modifications. The fate of m6A-modified mRNA depends on specific m6A readers that contribute to several processes such as mRNA splicing, degradation, and translation [8].

Previous studies have shown that m6A modifications are involved in important processes in cancer, including treatment resistance in hepatocellular carcinoma, breast cancer, and non-small-cell lung cancer [9–11]. Hao et al. recently suggested that ALKBH5 inhibited the progression of bladder cancer and sensitized bladder cancer cells to cisplatin through a casein kinase 2 α -mediated glycolysis pathway [12]. However, there is a lack of research about the role of m6A modification in EOC resistance to platinum. ALKBH5 was reported to be overexpressed in EOC tissues and promoted cancer progression by inhibiting EOC cell autophagy [13]. ALKBH5 was also suggested to contribute to resistance to PARP inhibitors in BRCA1/2-mutated ovarian cancer cells by regulating the expression of FZD10 mRNA and mediating the Wnt signaling pathway [14]. These data suggest that ALKBH5 plays a significant role in EOC and might be a potential therapeutic target.

Thus, this study aimed to explore the role of ALKBH5 and the underlying regulatory mechanism in platinum-resistant EOC.

2. Materials And Methods

2.1 Clinical sample collection

All patients signed informed consent before using clinical specimens. The use of specimens for this study has been proved by the ethics committee of the First Affiliated Hospital with Nanjing Medical University. Tumor tissues were obtained from patients who have undergone surgery in the First Affiliated Hospital with Nanjing Medical University (Jiangsu Province Hospital) between January 2015 to January 2019. Surgically resected specimens were immediately flash-frozen in liquid nitrogen for further investigation. According to National Comprehensive Cancer Network (NCCN) Guidelines, EOC patient who do not achieve complete clinical remission (CR) after initial platinum-based chemotherapy or tumor recurred within 6 months after CR is defined as a platinum-resistant case. Patients with a platinum-free interval longer than 6 months are defined as a platinum-sensitive case[15]. In our study, we obtained 15 platinum-sensitive samples and 9 platinum-resistant samples. All 24 patients underwent 6 courses of platinum-based adjuvant chemotherapy after surgery. Due to the strict surgical indication of recurrent EOC, the second operation of recurrent platinum-resistant EOC is not recommended. In the present study, platinum-resistant samples were obtained from patients who did not achieve CR or tumor recurred within 6 months after the first-line treatment. Meanwhile, different pathological types of EOC samples were also collected to investigate the ALKBH5 and HOXA10 expression (n = 32).

2.2 cell lines and culture conditions

The cell line HO8910, A2780, A2780-DDP, HO8910-DDP were cultured in RPMI1640 (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin at 37°C supplied with 5% CO₂. Among these cell lines, HO8910, A2780 are platinum-sensitive EOC cell lines. A2780 and HO8910 were continuous stepwise exposure to increasing concentration of cisplatin for constructing the cisplatin-resistant cell lines A2780-DDP and HO8910-DDP.

2.3 RNA extraction and qualified Real-time PCR (qPCR)

According to the manufacturer's instructions, total RNA was extracted from cultured cells and tissues with Trizol (Invitrogen). Then, cDNA was prepared by HiScript Q RT SuperMix for qPCR (Vazyme). The qPCR was performed with an SYBR Green PCR Kit (Vazyme). The sequences of gene primers used for qPCR were showed in **Supplementary Table 1**.

2.4 Protein extraction and Western blot assay

Total protein from cultured cells and tissues were lysed in RIPA buffer (Beyotime) with protease inhibitor (Beyotime) and then quantified by using a BCA assay kit (Beyotime). The quantified protein was separated by 10% SDS-PAGE and treated at 100°C for 5min. Western blot assays were performed as the protocol as we previously reported[16]. The antibodies used for western blot assay were listed in **Supplementary Table 2**.

2.5 Lentivirus infection and siRNA transfection

Lentiviral vectors pGC-FU-3FLAG-CBh-gcGFP-IRES-puromycin respectively encoding transcript ALKBH5 (oe-ALKBH5) and HOXA10 (oe-HOXA10) transcripts were purchased from Genechem (Shanghai, China), and cells were incubated with lentivirus and 4 mg/mL polybrene for 24 hours. Puromycin (0.5 µg/mL) was added to the medium for selection. All siRNA and plasmid were transfected in cells by using Lipofectamine 3000 (Invitrogen). Cell assays and RNA extraction were performed after 24h, and protein extractions were performed after 48h. All siRNAs were synthesized by GemmaPharma (Shanghai, China), and the down knocking efficiency was validated to select out the most effective target sequence. The selected target sequences of siRNA eventually used were showed in **Supplementary Table 3**

2.6 Proliferation assay

For Cell Counting Kit-8 (CCK8) assay, EOC cells were plated at a density of 6000 cells per well in 96-well plates. After cell adherence, cell proliferation was determined at 0, 24, 48, and 72 hours: CCK-8 (Vazyme) was added, and the plate was incubated at 37°C and 5% CO₂ for 1 h. Absorbance was measured at 450 nm on a microplate reader (TECAN)

For the EdU proliferation assay, EOC cells were plated at a density of 6000 cells per well in 96-well plates one day before treatment. Protocols were performed according to the of Cell-Light EdU Apollo567 In Vitro Kit (RiboBio).

2.7 Chemosensitivity assay

Lentivirus infected and siRNA transfected EOC cells were plated at a density of 6000–8000 cells per well in 96-well plates. A series of cisplatin (Sigma-Aldrich) concentrations (0, 5, 10, 15, and 20 μ M) were added. After 48 hours of treating, CCK8 was used to detect surviving cells. IC50 was graphically calculated by Graphpad 8.0.

2.8. Immunofluorescence(IF)assay

γ H2AX foci presents the degree of DNA double strand breaks, which could be used to evaluate cell DNA damage[17]. EOC cells were seeded in confocal dish and treated with cisplatin (5 μ M) for 6 hours. Cells were fixed in 4% paraformaldehyde and treated with 0.5% of Triton X-100 for 20 min and blocked in 1% Bovine Serum Albumin. Then cells were incubated with the first antibody in 4 $^{\circ}$ C overnight, and then incubated with the secondary antibody at room temperature for 1 h. The antibodies used for the IF assay were listed in **Supplementary Table 2**. Finally, cell nucleus was stained with DAPI (Merck). Images were captured by using Zeiss microscope.

2.9 Cell cycle assay and apoptosis assay

Cells were plated in 6-well plates, and respectively treated with cisplatin (5 μ M) and phosphate buffer saline (PBS) for 48h. For the cell cycle analysis, 1×10^6 cells and the cultural supernatant were collected, centrifuged, and washed. Cells were fixed with 75% cold ethanol for 24 h at -20 $^{\circ}$ C. Next, the fixed cells were stained in 500 μ l propidium oxide staining solution at room temperature for 15min in the dark. For the apoptosis assay, 2×10^4 cells and the cultural supernatant were collected. Then, 5 μ l of FITC Annexin V and 5 μ l of propidium iodide (BD Biopharmingen) were added to the collected cells and suspending in 300 μ l binding buffer for 15 min in the dark. Flow cytometry was performed to analyze cell cycle and cell apoptosis.

2.10 Immunohistochemistry (IHC) assay

Firstly, surgical samples were pretreated in 10% formaldehyde for fixed. Staining was performed by the protocol, as we previously reported[18]. The antibodies used for the IHC assay were listed in **Supplementary Table 2**.

2.11 RNA Binding Protein immunoprecipitation (RIP)-qPCR

The RIP assay was performed using a MagnaRIP RNA-Binding Protein Immunoprecipitation Kit (Millipore). Briefly, the cell lysates were incubated with beads coated with 5 μ g of antibodies with rotation at 4 $^{\circ}$ C overnight. Then, the RNA-protein-magnetic beads complexes were washed and eluted with proteinase K digestion buffer. Immunoprecipitated RNA was finally extracted by phenol-chloroform RNA extraction methods. Finally, enriched RNA was determined by qPCR and normalized to the input. The antibodies used for the RIP assay were listed in **Supplementary Table 2**.

2.12 Chromatin immunoprecipitation (ChIP)-qPCR

The ChIP assay was performed using a Chromatin Immunoprecipitation Kit (Millipore) according to the manufacturer's instructions. EOC cells were cross-linked with 1% formaldehyde; quenched with glycine at room temperature. Then, cells were collected, washed, and resuspended in lysis buffer. Then cross-linked DNAs were fragmented with 6% energy, 30s for 6 cycles. The sonicated chromatin solution was incubated with beads coated with 5 µg of antibodies with rotation at 4°C overnight. Immunoprecipitated DNA was purified and analyzed by qPCR and agarose gel electrophoresis assays. The specific primers were listed in **Supplementary Table 1**, and the antibodies used for ChIP assay were listed in **Supplementary Table 2**.

2.13 Luciferase reporter Assay.

The luciferase reporters respectively containing the coding sequence of wild

wild-type (WT) and mutated-type (Mut) of JAK2 3'UTR(Chr12:5126686–5127015) were synthesized by Genechem (Shanghai, China). Luciferase reporters, respectively containing the sequence of WT and Mut ALKBH5 promotor (Chr17:18181828–18181968) were synthesized by Tsingke (Nanjing, China). Cells were seeded in a 24-well plate, and respectively transfected with the WT/Mut reporters. Luciferase assay was performed with Luciferase Kit (Promega) under the manufacturer's instructions. The luciferase activity was measured by BERTHOL chemiluminescence measuring instrument (Centro XS LB 960). The sequences of the plasmids in the luciferase reporter assay were shown in **Supplementary Table 3**.

2.14 Animal studies

The animal studies were performed in accordance with the institutional ethics guidelines for animal experiments approved by the animal management committee of Nanjing Medical University. About 5×10^6 cells were injected subcutaneously into the axilla of the female athymic BALB/C nude mice (4 week-old, 18–20 g). When the average tumor size reached approximately 100mm^3 (after one week), mice were then randomized into two groups and treated with cisplatin (5mg/kg) or normal saline (NS) weekly. Tumor width (W) and length (L) was measured every week, and the volume (V) of the tumor was calculated by the formula $V = (W^2 \times L)/2$. Every group was treated by 6 cycles of cisplatin/NS, and mice were euthanized, and tumors were removed for further study.

2.15 Dot blot assay

mRNA was isolated from total RNA under the protocols of Kit (Promega). The dot blot assay was performed according to the bio-protocol database (<https://en.bio-protocol.org/e2095>). Briefly, 150/300 ng of isolated mRNA was then spotted onto a Hybond-N + membrane and cross-linked by a UV cross-linker. Methylene blue was used to interact with mRNA, and as the loading control, images were acquired. After washing, the membrane was washed and incubated first in blocking buffer and then with an anti-m6A antibody (1:250) overnight at 4°C. Then, the membrane was rewashed and incubated with an anti-rabbit antibody (1;10000). Eventually, the membrane was exposed to Hyperfilm ECL(Bio-Rad), and images were acquired. The antibodies used for dot blot assay were listed in **Supplementary Table 2**.

2.16 Actinomycin-D(Act-D) assay

The cells were plated in 6-well plates and treated with actinomycin D (5 µg/mL, Med Chem Express) respectively for 0, 2, 4, and 6 h. Total RNA was then extracted and quantified by qPCR. The gene expression at the indicated time was calculated and normalized by GAPDH.. The degradation rate of mRNA was estimated by the linear analysis.

2.17 m6A-modified RNA immunoprecipitation sequencing (MeRIP-seq) and MeRIP-qPCR

Intact mRNA was first isolated from total RNA samples using mRNA Isolation Kit according to the manufacturer's protocol (Promega), and the amount of purified mRNA was greater than 5µg. The Magna MeRIP™ m6A Kit (Millipore) was then used for MeRIP according to the manufacturer's instructions. Briefly, the isolated mRNA was chemically fragmented into 200-nucleotide-long fragments by incubation at 94°C for 5 min, and the size of the fragmented mRNA was confirmed by Agilent 2100 Bioanalyzer (Agilent, CA, USA). Then, m6A-methylated mRNAs were immunoprecipitated with the m6A-antibody (supplied by the kit). The major procedures included immunoprecipitation, washing, and elution. Then eluted RNA and MeRIPed RNA were analyzed by deep sequencing on an Illumina Novaseq™ 6000 platform at the LC-BIO Bio-techltd (Hangzhou, China) following the vendor's recommended protocol. The immunoprecipitated samples were also analyzed by MeRIP-qPCR. The specific primers are provided in **Supplementary Table 1**.

2.18 Statistical analysis

All data and error bars are presented as the mean ± SDs from at least three independent experiments. The two-tailed Student's t-test were performed to evaluate differences between two independent groups. The Graphpad 8.0 software was used to analyze the data. The indicated P values (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001) were considered statistically significant.

3. Results

3.1 ALKBH5 promotes cell resistance to cisplatin in vivo and in vitro

ALKBH5 expression was higher in platinum-resistant EOC cell lines than that in platinum-sensitive EOC cells. (Fig. 1A). Further, ALKBH5 was upregulated in platinum-resistant EOC samples (**Supplementary Figs. 1A and 1B**). EdU and CCK8 assays showed that ALKBH5 overexpression significantly promoted cell proliferation (Fig. 1C and 1D), while the opposite effect was observed in ALKBH5 knockout by specific siRNAs (Fig. 1B, **Supplementary Fig. 1C-E**). The chemosensitivity assay showed an increased IC50 of cisplatin in EOC cells with ALKBH5 overexpression (Fig. 1E). In contrast, ALKBH5 knockdown weakened the chemoresistance in cisplatin-resistant EOC cells (**Supplementary Fig. 1G**).

Moreover, ALKBH5 overexpression could relieve the DNA damage induced by cisplatin, whereas ALKBH5 knockdown aggravated cisplatin-induced DNA damage (Fig. 1F and **Supplementary Fig. 1H**).

Furthermore, the cell cycle analysis showed that ALKBH5 overexpression significantly relieved the blocking effect in the G2/M phase induced by cisplatin (Fig. 1G). Apoptosis analysis also showed that ALKBH5 overexpression reduced cisplatin-induced cell apoptosis (Fig. 1H). Consistently, animal studies showed that ALKBH5 overexpression promoted EOC tumor growth and resistance to cisplatin in vivo (Fig. 1I).

3.2 ALKBH5-HOXA10 loop maintains ALKBH5 and HOXA10 overexpression in EOC

To explore the molecular mechanism of ALKBH5-mediated EOC resistance to cisplatin, RNA sequencing (RNA-seq) was performed. The results of functional annotations of RNA-seq via Gene Ontology (GO) analysis showed enhanced DNA repair in EOC cells with ALKBH5 upregulation (**Supplementary Fig. 2, Additional File 1**). Further, RNA-seq demonstrated that homeobox A10 (HOXA10) was highly upregulated in EOC cells with ALKBH5 overexpression ($\log_2FC = 2.52$). Our previous studies have demonstrated that HOXA10 influences EOC cell proliferation, metastasis, invasion, and differentiation[19–21]. RIP-qPCR assay indicated that HOXA10 mRNA was significantly enriched in Flag-specific antibody in EOC cells transfected with oe-ALKBH5 using Flag-tag, suggesting that HOXA10 mRNA could bind with ALKBH5 (Fig. 2A). According to its transcript expression change and the potential regulatory mechanism, we speculated that ALKBH5 might mediate HOXA10 upregulation by maintaining the stability of HOXA10 mRNA. Results of the Act-D assay indicated that the half-life period of HOXA10 mRNA was significantly increased with ALKBH5 overexpression (Fig. 2B).

Analysis of the correlation between ALKBH5 and HOXA10 in 32 EOC tissues in The Cancer Genome Atlas (TCGA) database showed that mRNA expression of HOXA10 was positively correlated with ALKBH5 expression (**Supplementary Fig. 3A**). Consistently, HOXA10 expression was found to be regulated by ALKBH5 upregulation or downregulation in EOC cells (Fig. 2C and 2D). Considering the role of HOXA10 as a transcription factor (TF), we analyzed data from the JASPAR database and unexpectedly found probable binding motifs with HOXA10 in the ALKBH5 promoter. The sonicated chromatin solution of EOC cells was immunoprecipitated with an anti-Flag antibody and IgG control antibody. Results of the ChIP-qPCR showed that the Flag-specific antibody was significantly enriched in DNA fragments contained in the ALKBH5 promoter region (Fig. 2E). By validating the potential transcription binding motifs of HOXA10 and ALKBH5 predicted by the JASPAR database, we constructed luciferase reporting gene plasmids encoding WT and Mut DNA sequences of ALKBH5 promoter regions. The luciferase reporter assay results suggested that HOXA10 could be a TF to interact with the TAAA region of the ALKBH5 promoter (Fig. 2F). Consequently, ALKBH5 expression was confirmed to be regulated by HOXA10 in EOC cells (Fig. 2G-J). These data suggested that HOXA10 might play as an upstream TF of ALKBH5 and contribute to the upregulation of ALKBH5 in EOC. Our findings revealed that the ALKBH5-HOXA10 regulation loop steadily maintained the overexpression of both ALKBH5 and HOXA10 in EOC.

3.3 HOXA10 overexpression promotes the proliferation and cisplatin-resistance of EOC in vivo and in vitro

Given that ALKBH5 was confirmed to promote EOC resistance to cisplatin and form a positive regulation loop with HOXA10, we speculated that HOXA10 might also promote platinum resistance in EOC. The results showed that HOXA10 expression was upregulated in A2780-DDP and HO8910-DDP cells (Fig. 3A). Additionally, HOXA10 was confirmed to be overexpressed in platinum-resistant EOC samples (**Supplementary Fig. 3B and 3C**). This confirmed that HOXA10 overexpression significantly promoted cell proliferation (Fig. 3B and 3C), while an opposite effect is exerted after HOXA10 knockdown (**Supplementary Fig. 3D-F**). Further, HOXA10 overexpression promoted cisplatin resistance and attenuated the DNA damage in cisplatin-sensitive EOC cells (Fig. 3D and 3E). In contrast, HOXA10 knockdown weakened cisplatin resistance and aggravated DNA damage in cisplatin-resistant EOC cells (**Supplementary Fig. 3G-I**). In cell cycle analysis, HOXA10 overexpression significantly altered the inhibition effect of cisplatin in the G2/M phase (Fig. 3F). The apoptosis analysis results showed that HOXA10 overexpression inhibited EOC cell apoptosis induced by cisplatin (Fig. 3G). The xenograft model demonstrated that HOXA10 promoted tumor growth and resistance to cisplatin (Fig. 3H).

3.4 ALKBH5 erases m6A modifications of JAK2 mRNA and maintains JAK2 mRNA expression by lowering YTHDF2-mediated mRNA degradation

Given that ALKBH5 was identified as an m6A eraser, we performed an m6A dot blot assay and found that ALKBH5 upregulation in EOC cells decreased the m6A modification level of mRNA (**Supplementary Fig. 4A**). We further analyzed the targeted m6A-modified genes of ALKBH5 in EOC using RNA-Seq and MeRIP-Seq analysis. The results of MeRIP-Seq validated the differentially enriched m6A modification motif in the m6A-immunopurified RNA in A2780 cells (Fig. 4A). Further, 51.88% of the m6A abundance was differentially enriched in three prime untranslated regions (3'UTRs) of transcripts (Fig. 4B). MeRIP-seq revealed that ALKBH5 upregulation resulted in increased abundance of m6A peaks in 586 transcripts and decreased abundance in 792 transcripts ($|\log_2 \text{fold change (FC)}| \geq 1, P \leq 0.05$) (Fig. 4C and **Additional File 2**). RNA-seq also revealed that there were 2005 upregulated and 676 downregulated genes in A2780 with ALKBH5 upregulation ($|\log_2 \text{FC}| \geq 1, P \leq 0.05$) (Fig. 4C and **Additional File 3**). The quadrant diagram graph displayed transcripts with a different abundance of m6A peaks and regulated gene expression (Fig. 4D). ALKBH5 mediated m6A demethylation of mRNA, which could influence mRNA metabolism processes such as stabilization and degradation. Thus, we focused on genes with both changes in m6A modification and mRNA expression. There were 19 genes that not only exhibited an obviously decreased abundance of m6A peaks ($\log_2 \text{FC} \leq -2, P < 0.05$), but also significantly discrepant expression regulation ($\log_2 \text{FC} \geq 2, P < 0.05$) (Fig. 4E and Table 1). This suggested that these 19 genes might be contained in the target m6-modified genes regulated by ALKBH5. Among them, m6A abundance in the 3'UTR region of JAK2 mRNA (Chr12:5126686–5127015) was notably significantly decreased after ALKBH5 upregulation in A2780 ($\log_2 \text{FC} = -2, P = 0.03$) (Fig. 4F). The JAK2/STAT3 signaling pathway has been widely demonstrated to be involved in tumor growth and chemoresistance in various malignancies [22]. Thus, we speculated that ALKBH5 overexpression might promote EOC cell proliferation and resistance to cisplatin by targeting JAK2 and activating the JAK2/STAT3 signaling pathway in an m6A-dependent manner. The results of MeRIP-qPCR also confirmed that ALKBH5 overexpression decreased the m6A modification abundance of the 3'UTR region of JAK2 mRNA in A2780 (Fig. 4G). The results of RIP-qPCR

assay showed that JAK2 mRNA expression was higher in the Flag-specific antibody than that in the IgG antibody (Fig. 4H). We then performed the Act-D assay to investigate whether ALKBH5 expression affected the stability of JAK2 mRNA. As shown in Fig. 4I, JAK2 mRNA expression was highly stable in EOC cells with ALKBH5 overexpression. The distribution of m6A is usually embedded within the consensus sequence 5'-DRACH' (D = G/A/U, R = A/G, H = not G). Based on the results of MeRIP-seq, we mutated three "DRACH" motifs of JAK2 3'UTR (Chr12:5126686–5127015) to construct the WT/Mut luciferase reporter. Moreover, results of the luciferase reporter assay showed that ALKBH5 bound with the m6A-motif in the 3'UTR region of JAK2 mRNA and promoted *JAK2* expression (Fig. 4J). The correlation analysis supported that JAK2 mRNA expression was also positively correlated with ALKBH5 expression in EOC tissues (**Supplementary Fig. 4B**) and that ALKBH5 regulated JAK2 mRNA expression in EOC cells (Fig. 4K and 4L). Previous studies have identified YTHDFs, including YTHDF1/2/3, as a family of m6A readers that target thousands of mRNA transcripts by distinctly recognizing the m6A motif. In the cytosol, YTHDF1 enhances the translation of its targets by interacting with initiation factors and facilitating ribosome loading. YTHDF3 affects the translation of its target mRNAs along with YTHDF1. Meanwhile, YTHDF2 helps promote mRNA degradation of m6A-modified mRNA [23]. In the present study, we found a lower m6A abundance of JAK2 mRNA in EOC cell with ALKBH5 overexpression, whereas JAK2 mRNA expression was upregulated. Considering the results above, we speculated that YTHDF2 might mediate m6A-JAK2 degradation. The RIP-qPCR assay showed that the YTHDF2-specific antibody caused a significantly higher increase in JAK2 mRNA enrichment than did IgG (Fig. 4M). The Act-D assay confirmed that the level of JAK2 mRNA was more stable after YTHDF2 knockdown (Fig. 4N). We then used the specific siRNA to knockdown YTHDF2 expression in EOC cells. The results showed that YTHDF2 knockdown caused JAK2 expression to be higher than that in the negative control. Meanwhile, YTHDF2 knockdown reduced JAK2 expression in cells with ALKBH5 overexpression (Fig. 4O and 4P). Collectively, these findings indicated that ALKBH5-mediated JAK2 mRNA m6A demethylation inhibited YTHDF2-mediated mRNA degradation and maintained JAK2 mRNA expression.

Table 1

Gene name	RNA-seq		MeRIP-seq	
	diff. p	diff. log2fc	diff. p	diff. log2fc
NHLRC2	0.002	-2.83	0.001	2.49
SLC2A13	0.006	-2.16	0.001	2.50
TFPI	0.009	-2.09	0.000	2.72
SETDB2	0.009	-2.54	0.000	2.95
VCAN	0.012	-2.23	0.000	3.50
CPLANE1	0.018	-2.18	0.000	4.00
CP	0.019	-2.86	0.000	3.88
KIF27	0.019	-2.95	0.000	3.37
CEP290	0.024	-2.15	0.000	4.42
GAB2	0.025	-3.17	0.002	2.40
DST	0.025	-2.93	0.000	4.01
HLA-L	0.027	-2.92	0.005	2.23
PHIP	0.028	-2.20	0.000	4.38
SP100	0.030	-2.03	0.002	2.34
JAK2	0.035	-2	0.000	3.35
DOP1A	0.036	-2.12	0.000	2.74
ATAD2B	0.037	-2.76	0.008	2.04
HLA-L	0.045	-2.63	0.005	2.23
VCAN	0.047	-2.65	0.000	3.50
LRR37A3	0.047	-2.87	0.007	2.17
RSF1	0.048	-2.14	0.000	3.43

In total 19 genes were found exhibited obvious decreased abundance of m6A peaks ($\log_2(\text{FC}) \leq -2$, $P \leq 0.05$), and displayed significantly discrepant expression regulation ($\log_2(\text{FC}) \geq 2$, $P \leq 0.05$).

3.5 ALKBH5-HOXA10 loop promotes resistance to cisplatin by activating the JAK2/STAT3 signaling pathway in EOC

We then investigated whether HOXA10 or ALKBH5 knockdown could rescue cisplatin resistance in EOC cells overexpressing ALKBH5 and HOXA10. siHOXA10 transfection in cells with ALKBH5 overexpression

showed that HOXA10 knockdown could partly rescue EOC cell proliferation, chemoresistance, and DNA damage response (DDR) induced by ALKBH5 overexpression (Fig. 5A-D). Similarly, ALKBH5 knockdown in cells with HOXA10 overexpression also partly relieved EOC cell proliferation, chemoresistance, and DDR (Fig. 5A-D). Further analysis confirmed that upregulation of the ALKBH5-HOXA10 loop could promote JAK2 expression and the phosphorylation level of STAT3, which represents activation of the JAK2/STAT3 signaling pathway (Fig. 6A). In contrast, knocking down ALKBH5 or HOXA10 decreased JAK2 expression and the phosphorylation level of STAT3, which indicates inhibition of the JAK2/STAT3 signaling pathway (Fig. 6B). Moreover, the activated JAK2/STAT3 pathway could be rescued by siHOXA10 and siALKBH5 in EOC cells with HOXA10 and ALKBH5 overexpression, respectively (Fig. 6C and 6D).

Immunohistochemistry (IHC) assay of the xenograft tissues showed the same results (Fig. 6E and 6F). Collectively, these results support that JAK2/STAT3 signaling pathway might be involved in the regulation mechanism of the ALKBH5-HOXA10 loop in EOC. Furthermore, WP1066, an inhibitor of the JAK2/STAT3 signaling pathway, effectively suppressed cancer cell proliferation, resistance to cisplatin, and DDR induced by ALKBH5 and HOXA10 overexpression in EOC cells (**Supplementary Fig. 5**). Overall, these findings indicate that consistent overexpression of the ALKBH5-HOXA10 loop in EOC could promote tumor growth and chemoresistance by mediating the JAK2/STAT3 signaling pathway.

4. Discussion

The role of m6A-modification in treatment resistance in cancer, particularly to platinum chemotherapy in EOC, remains unclear. The present study found that ALKBH5 is upregulated in platinum-resistant EOC, and this promotes cell proliferation and chemoresistance in vivo and vitro. Based on the results of RNA-seq, we identified the ALKBH5-HOXA10 positive regulation loop and then confirmed that HOXA10 could also promote cell proliferation and chemoresistance in vivo and vitro. The results of MeRIP-seq support that ALKBH5 reduced m6A modification in JAK2 mRNA and maintain JAK2 mRNA expression by reducing YTHDF2-mediated mRNA degradation. Furthermore, our findings revealed that overexpression of the ALKBH5-HOXA10 loop activates the JAK2/STAT3 signaling pathway, and this, in turn, promotes platinum resistance in EOC (Fig. 7).

As one of the early adaptive mechanisms by which cells respond to environmental stress, m6A modification appears to be a promising target for cancer treatment. Previous studies have demonstrated the important roles of m6A-modulators in EOC, including those of MATTTL3, ALKBH5, FTO, YTHDF1, and IGF2BP1[24–28]. These studies suggest that m6A modification significantly contributes to EOC initiation and progression. However, research on the role of m6A modification in EOC chemoresistance is lacking.

In recent years, ALKBH5 has been increasingly studied in various malignancies and has been found to play dual roles. Previous studies demonstrated that ALKBH5 acted as an oncogene in colon cancer, endometrial cancer, and renal cell carcinoma [29–31]. Further, ALKBH5 was found to suppress progression in pancreatic cancer and hepatocellular carcinoma[32, 33]. Moreover, ALKBH5 was also suggested to increase glioma stem cell radioresistance by regulating homologous recombination[34]. Meanwhile, Li et al. identified ALKBH5 to enhance treatment response to anti-PD-1 therapy in melanoma,

colorectal, and potentially other cancers[35]. These studies indicated that ALKBH5 might be a potential target to overcome treatment resistance in cancer. Similarly, our findings confirmed that ALKBH5 was an oncogene in EOC [36]. In the present study, we discovered that ALKBH5 was upregulated in platinum-resistant EOC and promoted cell resistance to cisplatin. Functional annotations based on the results of RNA-seq suggested that ALKBH5 upregulation was associated with DNA repair processes in cancer cells. Subsequently, we found that γ H2AX expression was significantly decreased in EOC cells with ALKBH5 overexpression. This suggested that ALKBH5 could enhance DNA repairs of DNA double-strand break induced by cisplatin. To our best knowledge, this study is the first to report ALKBH5 upregulation in platinum-resistant EOC and the consequent promotion of cell resistance from such upregulation.

HOXA10 is a member of the homeobox gene family that acts as a TF in embryonic development. Aberrant regulation of the homeobox family has been identified in various cancers[37–39]. Our previous studies have determined the critical role of HOXA10 in ovarian cancer [40–42], and we have been exploring the role of HOXA10 in female malignancies for a long time. However, studies on the role of HOXA10 in platinum-resistant EOC are lacking. The results of RNA-seq highlighted the importance of HOXA10, and further analysis revealed its crucial role in facilitating chemoresistance in EOC. Moreover, we identified ALKBH5-HOXA10 as a positive feedback loop that could steadily maintain each other's upregulation. HOXA10 binding on the "TAAA" region of ALKBH5 promotor enhanced ALKBH5 transcription. Meanwhile, ALKBH5 could interact with HOXA10 mRNA and maintain its stabilization.

JAK2 functions as a prototypical kinase that phosphorylates STAT3, which activates the JAK2/STAT3 signaling pathway and promotes tumorigenesis and progression in several cancer types [43]. Activation of the JAK2/STAT3 signaling pathway was also proven to contribute to chemotherapy resistance in several malignancies, including in EOC [44–46]. The m6A-dependent regulation of SOCS3 and JAK2 mediated by YTHDF1/YTHDF2 has been demonstrated in pluripotent stem cells [47]. However, to our best knowledge, no study has reported ALKBH5-mediated m6A demethylation of JAK2 to date. Based on the results of MeRIP-seq, we found that ALKBH5 erased the m6A-modification of JAK2 mRNA 3'UTR. Thus, we further investigated the probable reader that mediated m6A-JAK2 mRNA metabolism and found that YTHDF2 could directly bind with JAK2 mRNA and interact with its m6A motif, which prevents the degradation of demethylated JAK2 mRNA. YTHDF2 is a well-established m6A reader that can weaken mRNA stability by interacting with m6A-containing mRNAs [48]. These findings suggested that ALKBH5 mediates JAK2 m6A demethylation in EOC cells, which maintains JAK2 mRNA expression by lowering YTHDF2-mediated mRNA degradation.

This study has some limitations. As a well-known m6A demethyltransferase, ALKBH5 usually regulates the target gene expression in an m6A-dependent manner. Our study demonstrated that ALKBH5 could bind with HOXA10 and maintain mRNA expression. However, the results of MeRIP-seq showed that there was no differentially enriched m6A "peak" in HOXA10 mRNA after ALKBH5 overexpression. These findings suggest that ALKBH5 does not influence HOXA10 in a direct m6A-dependent manner. Thus, the exact molecular mechanism needs further investigations.

In summary, our findings revealed that m6A modification represents a novel mechanism of cisplatin resistance in EOC. ALKBH5 was upregulated in platinum-resistant EOC, and ALKBH5 overexpression promoted EOC cell proliferation and resistance to cisplatin in vivo and in vitro. HOXA10 was identified as a TF enhancing ALKBH5 transcription, and it could also be regulated by ALKBH5. Further, HOXA10 was found to play a role in EOC resistance to platinum. Further analysis of the m6A modification mechanism regulated by ALKBH5 showed that JAK2 is the m6A-modified gene targeted by ALKBH5. ALKBH5 overexpression maintained JAK2 mRNA stability in a YTHDF2-mediated manner. Consistent upregulation of the ALKBH5-HOXA10 loop promoted EOC tumor growth and resistance to cisplatin by activating the JAK2/STAT3 signaling pathway in an m6A-dependent manner. Collectively, our results suggest that inhibition of the expression of the ALKBH5-HOXA10 loop represents a potential strategy to overcome platinum resistance in EOC.

Declarations

Ethics approval and consent to participate

All patients signed informed consent before using clinical specimens. The use of specimens for this study has been proved by the ethics committee of the First Affiliated Hospital with Nanjing Medical University. The animal studies were performed in accordance with the institutional ethics guidelines for animal experiments approved by the animal management committee of Nanjing Medical University.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions:

Conceptualization, Wenjun Cheng, Sipei Nie and Lin Zhang; Methodology, Sipei Nie, Lin Zhang and Yicong Wan; Bioinformatics Analysis, Jinhui Liu; Writing -Original Draft, Sipei Nie, Jing Yang and Rui sun; Writing -Review & Editing, Lin Zhang, Wenjun Cheng; Funding Acquisition, Wenjun Cheng and Yijiang;

Supervision, Guodong Sun, Xiaolin Ma and Hui Wang. All authors read and approved the final manuscript.

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Sipei Nie, Lin Zhang, Jinhui Liu and Yicong Wan contributed equally to this study.

Abbreviations

3'-UTR: three prime untranslated region; Actinomycin-D (Act-D); Agarose gel electrophoresis (AGE); AlkB homolog 5 (ALKBH5); Cell Counting Kit-8 (CCK8); Chromatin immunoprecipitation (ChIP); clinical remission (CR); DNA damage repairment (DDR); epithelial ovarian cancer (EOC); fat mass and obesity associated (FTO); Homeobox A10 (HOXA10); (HOXA10) Immunofluorescence (IF); Immunohistochemistry (IHC); insulin-like growth factor-2 mRNA binding proteins (IGF2BP1–3), N6-methyladenosinemethyltransferase-like (m6A); 3 (METTL3), methyltransferase-like14 (METTL14); m6A-modified RNA immunoprecipitation sequencing (MeRIP-seq); messenger RNA (mRNA); mutated-type (Mut); National Comprehensive Cancer Network (NCCN); normal saline (NS) phosphate buffer saline (PBS); RNA immunoprecipitation (RIP), RNA sequencing (RNA-seq), transcription factor (TF), qualified Real-time PCR (qPCR); wide-type (WT); WT1 associated protein (WTAP)

YTH domain family (YTHDF).

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Figures

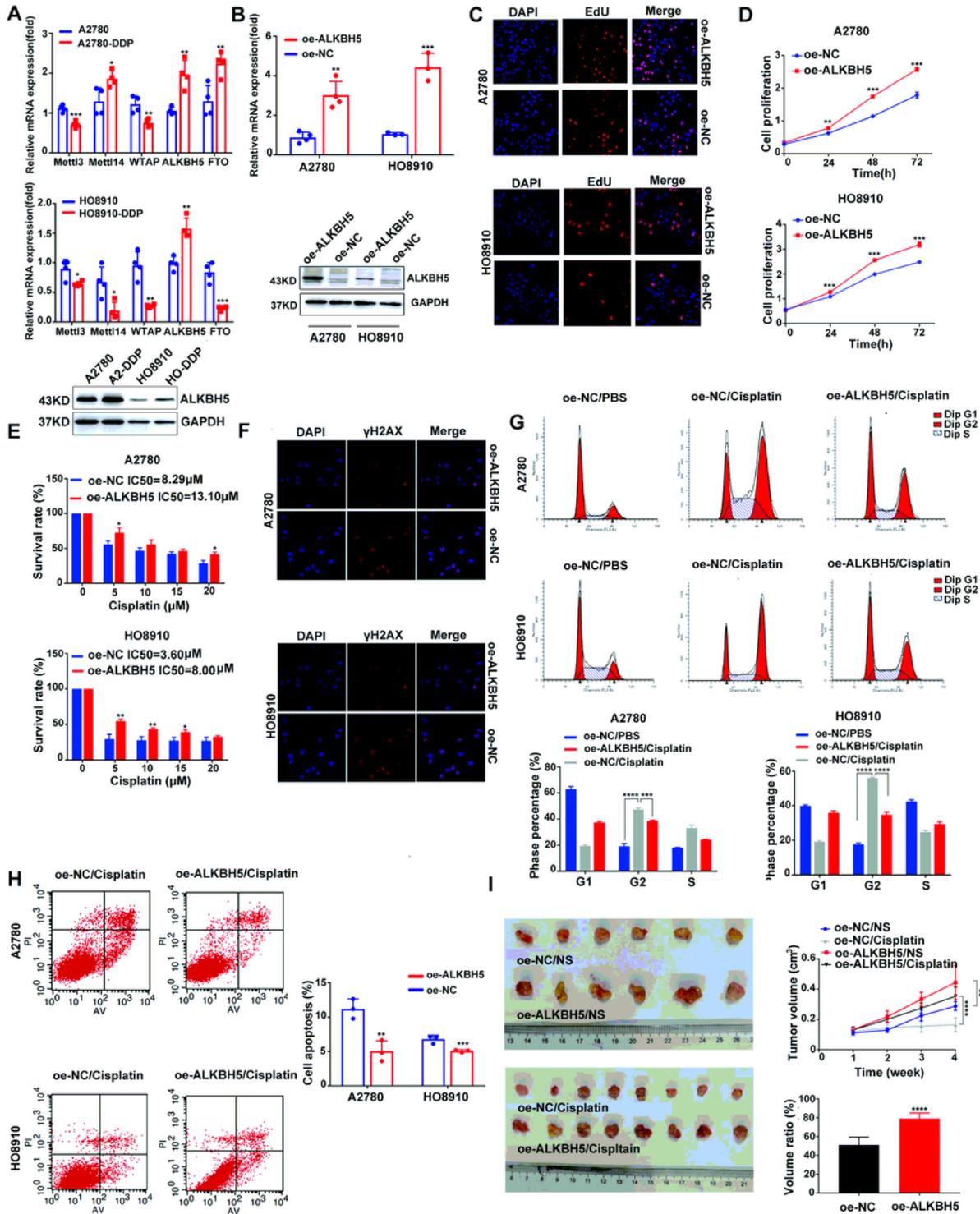


Figure 1

ALKBH5 overexpression promotes EOC cell proliferation and resistance to cisplatin in vitro and in vivo (A) The expression of m6A modifiers is investigated in cisplatin-sensitive and cisplatin-resistant EOC cells, and ALKBH5 is found to be upregulated in cisplatin-resistant EOC cells. (B) The transfection efficiency of

oe-ALKBH5 lentivirus in A2780 and H08910. (C and D) The EdU proliferation assay and CCK8 assay confirm that ALKBH5 overexpression promotes cell proliferation in EOC cells. (E) The chemosensitivity assay shows that the IC50 of cisplatin is higher in EOC cells with ALKBH5 overexpression. (F) The IF assay shows that γ H2AX expression is reduced in EOC cells with ALKBH5 overexpression after cisplatin treatment (5 μ M, 6h). (G) The cell cycle assays show that ALKBH5 overexpression can attenuate blocking in the G2/M phase induced by cisplatin (5 μ M, 48h). (H) The apoptosis assay shows that ALKBH5 overexpression can decrease the percentage of cell apoptosis induced by cisplatin (5 μ M, 48h). (I) The animal study shows that ALKBH5 overexpression promotes tumor growth and chemoresistance to cisplatin in vivo.

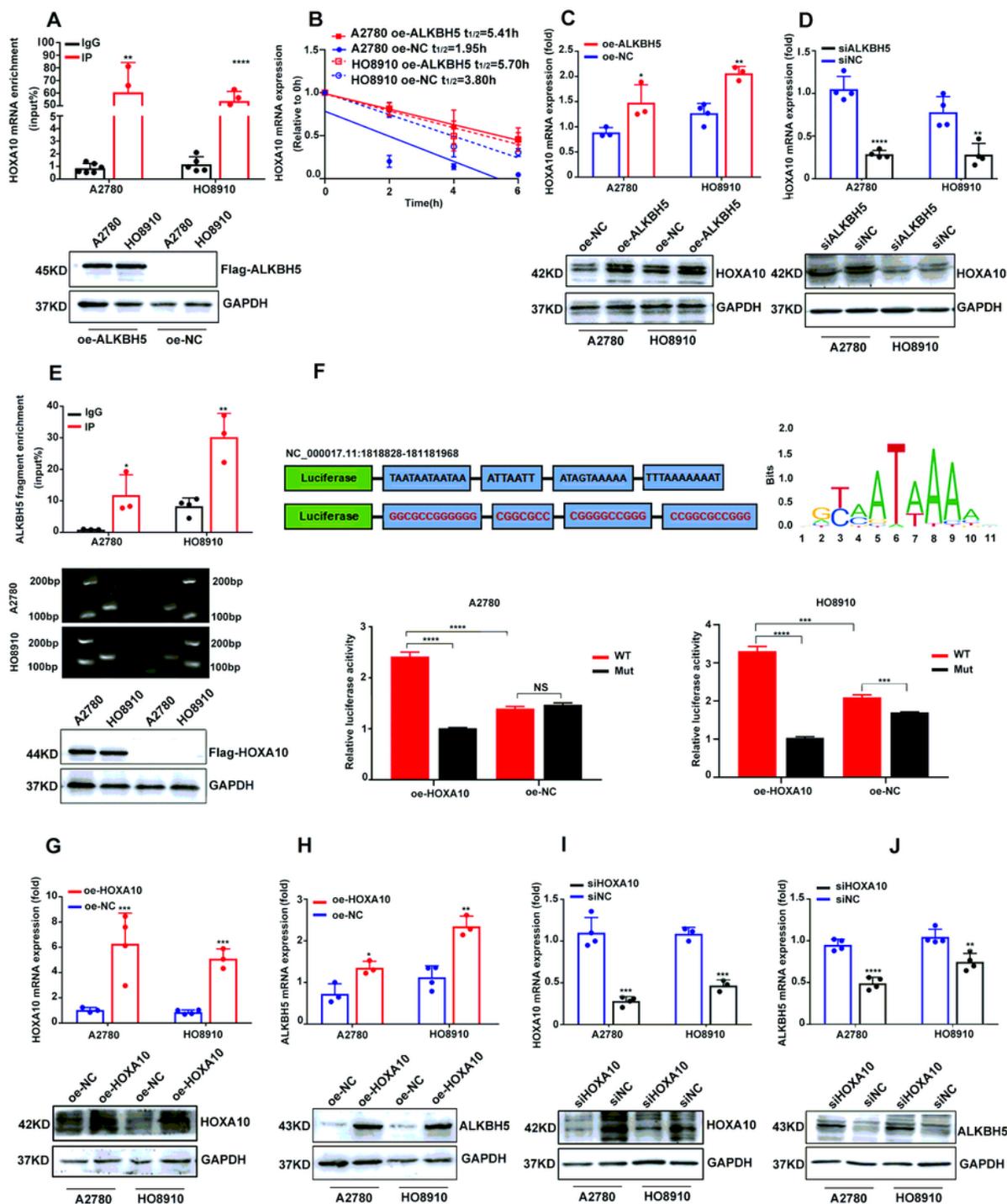


Figure 2

ALKBH5-HOXA10 loop maintains ALKBH5 and HOXA10 overexpression in EOC (A) The results of the RIP-qPCR assay show that HOXA10 mRNA is enriched in Flag-specific antibody in EOC cells on transfection with oe-ALKBH5 using Flag-tag. (B) The Act-D assay shows that ALKBH5 overexpression can maintain HOXA10 mRNA stability in EOC cells. (C and D) ALKBH5 regulates HOXA10 expression in EOC. (E) The ChIP-qPCR assay suggests that the ALKBH5 promoter fragment is enriched in HOXA10 (top), and the AGE

assay validates the sonicated products and immunoprecipitated DNA of the ChIP assay (middle). EOC cells are transfected with HOXA10-Flag-tag (bottom). (F) Based on the TF binding motif of HOXA10 predicted by the JASPAR database (top), the luciferase reporter assay is conducted, and the results show that HOXA10 could interact with the region containing TAAA of ALKBH5 promoter (bottom). (G-J) HOXA10 regulated ALKBH5 expression.

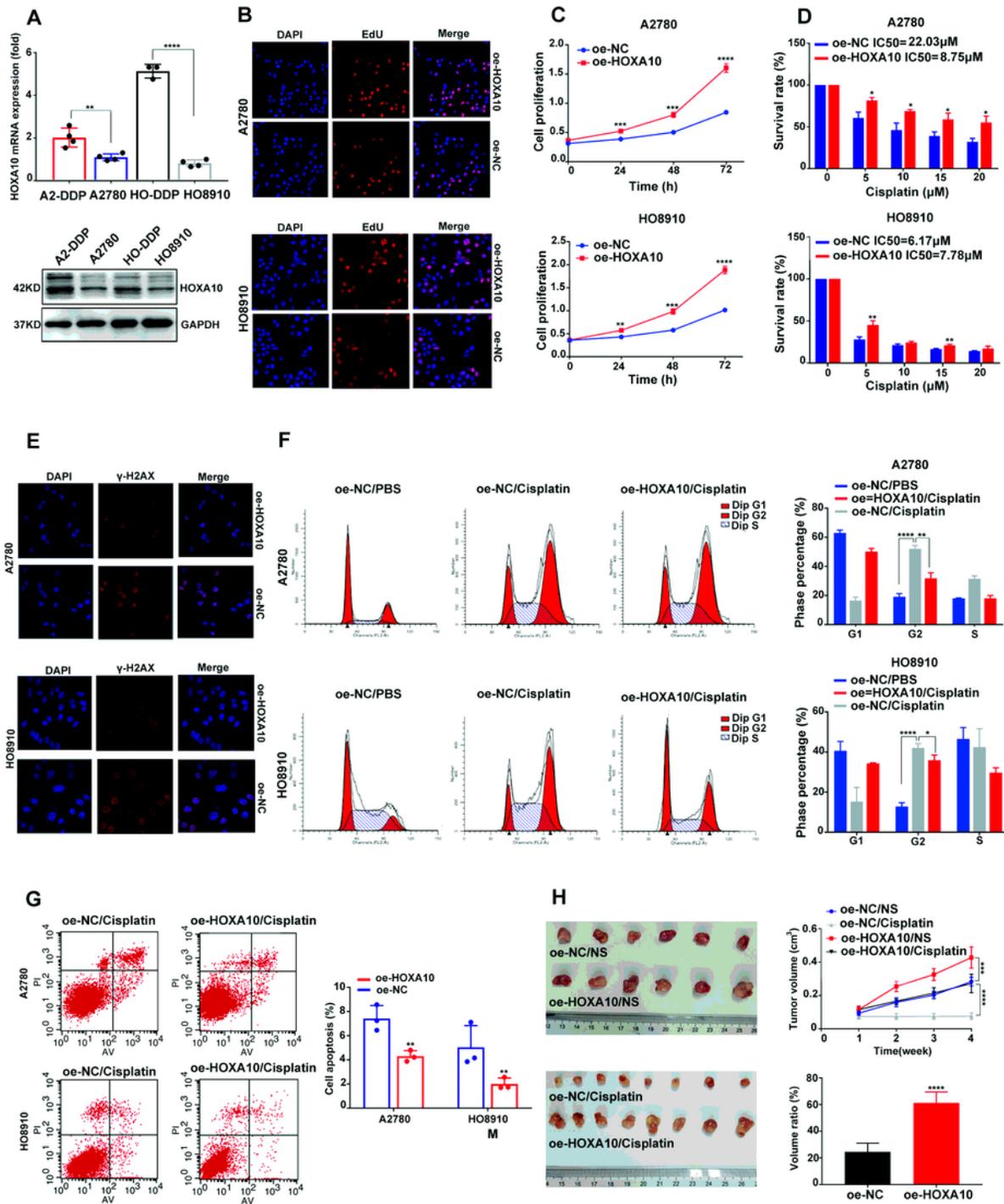


Figure 3

HOXA10 overexpression promotes EOC cell proliferation and cisplatin resistance in vitro and vivo (A) HOXA10 was verified to be upregulated in cisplatin-resistant EOC cells. (B and C) The EdU proliferation assay and CCK8 assay confirm that ALKBH5 overexpression promotes cell proliferation in EOC cells. (D) The chemosensitivity assay demonstrates higher IC50 of cisplatin in EOC cells with oe-HOXA10 transfection. (E) The IF assay shows that γ H2AX expression is decreased in EOC cells with oe-HOXA10 transfection after cisplatin treatment (5 μ M, 6h). (F) The cell cycle assays show that HOXA10 overexpression can alleviate the blocking in the G2/M phase induced by cisplatin (5 μ M 48h). (G) The apoptosis assays show that HOXA10 overexpression can lower the percentage of cell apoptosis induced by cisplatin (5 μ M, 48h). (H) The animal study shows that HOXA10 overexpression promotes cell proliferation and resistance to cisplatin in vivo.

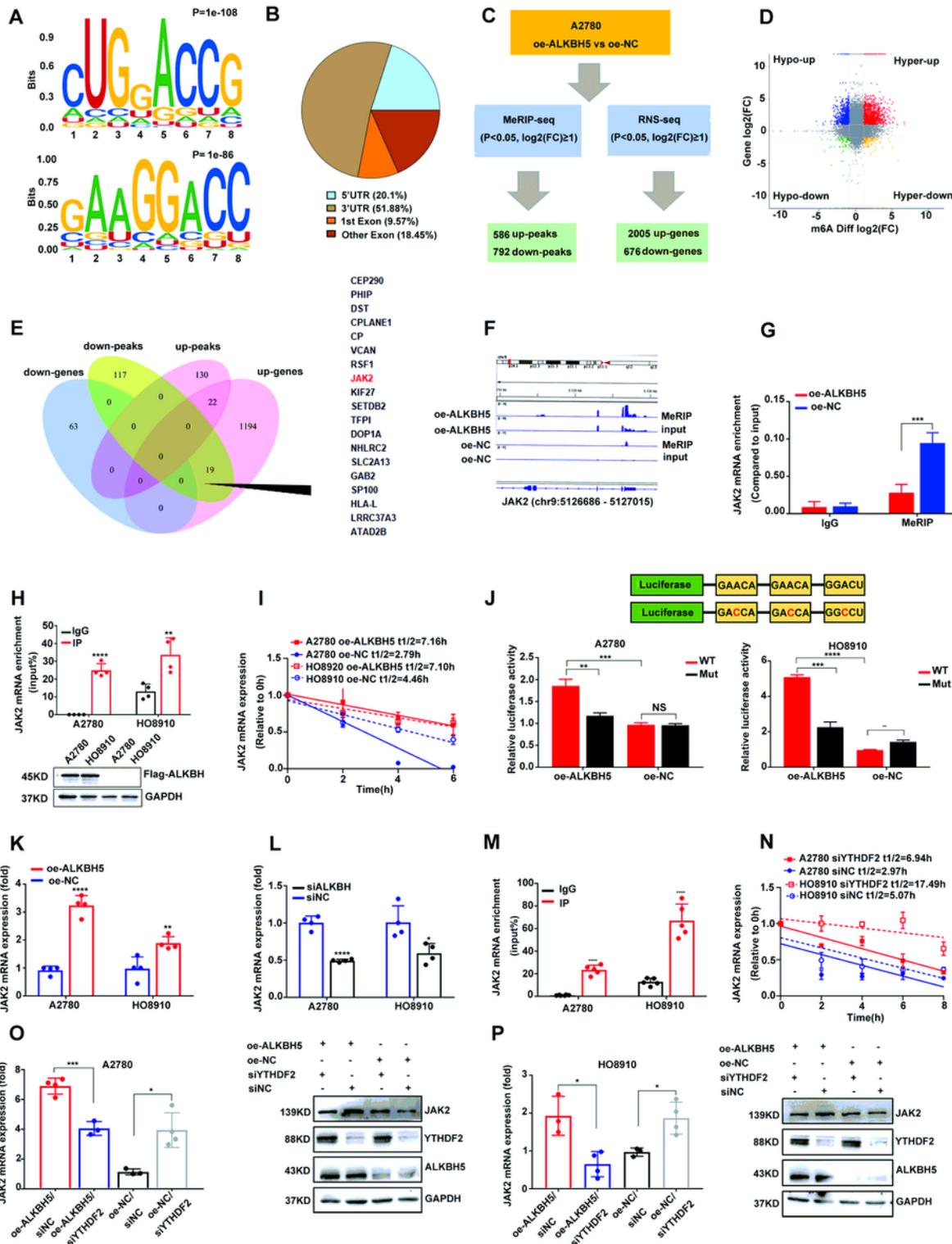


Figure 4

ALKBH5 erases m6A modifications of JAK2 mRNA and maintains JAK2 mRNA expression by lowering YTHDF2-mediated mRNA degradation (A) The two differentially enriched m6A-modification motif in the immunopurified RNA in A2780. (B) Distribution of regulated m6A peaks in mRNA is detected by MeRIP-seq after ALKBH5 overexpression. (C) Schematic of downstream analysis for MeRIP-Seq and mRNA-seq. (D) The quadrant diagram graph displays the transcripts with different m6A peaks and regulated gene

expression based on MeRIP-seq and RNA-seq. (E) The Venn diagram shows the genes detected by MeRIP-seq and RNA-seq; the 19 candidate target genes of ALKBH5 are shown on the right. (F) m6A abundances in JAK2 mRNA transcripts in cells with ALKBH5 overexpression (MeRIP and input) and in negative control (MeRIP and input). m6A regulation is calculated as the ratio of m6A abundances of MeRIP to input ($\log_2FC=-2$, $P=0.03$). (G) MeRIP-qPCR confirms that ALKBH5 upregulates the m6A peak in the 3' untranslated region of JAK2 mRNA. (H) RIP-PCR confirmed ALKBH5 binding to JAK2 mRNA. (I) The act-D assay shows an increased lifespan of JAK2 mRNA after ALKBH5 overexpression. (J) Relative luciferase activity of the wild-type or mutant JAK2 3'UTR luciferase reporter in EOC cells with ALKBH5 overexpression and the negative control. (K and L) ALKBH5 regulates JAK2 mRNA expression. (M) RIP-qPCR confirms YTHDF2 binding to JAK2 mRNA. (N) Increased lifespan of JAK2 mRNA after YTHDF2 silencing. (O and P) YTHDF2 remarkably regulates JAK2 expression in A2780 and H08910 cell lines.

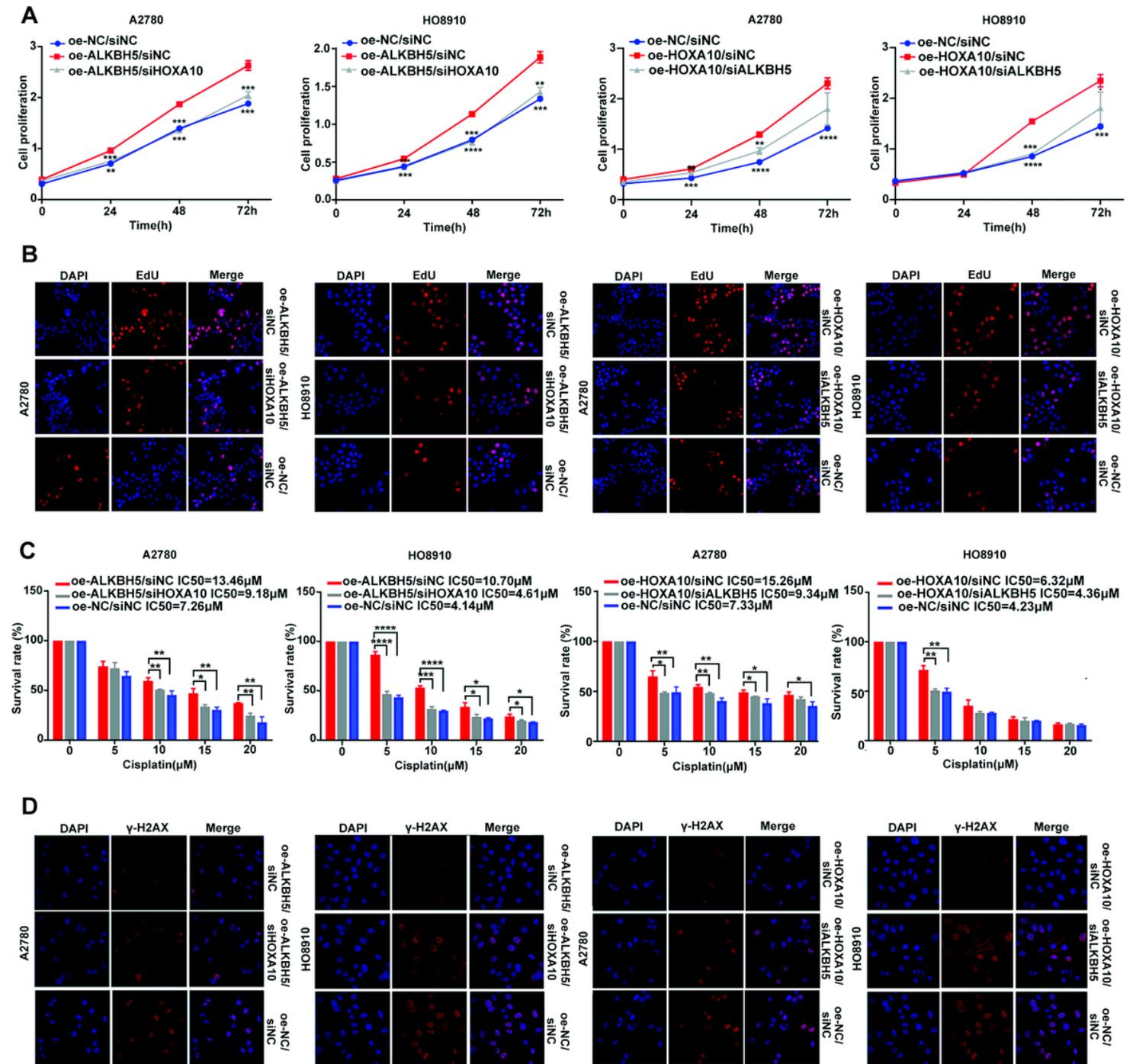


Figure 5

ALKBH5-HOXA10 loop steadily promotes cell proliferation and resistance to cisplatin (A and B) The EdU and CCK8 proliferation assays show that HOXA10 (left) or ALKBH5 (right) knockdown in EOC cells with ALKBH5 or HOXA10 expression suppresses cancer cell proliferation. (C and D) HOXA10 (left) or ALKBH5 (right) knockdown rescues resistance to cisplatin and DDR in EOC cells with ALKBH5- HOXA10 loop overexpression.

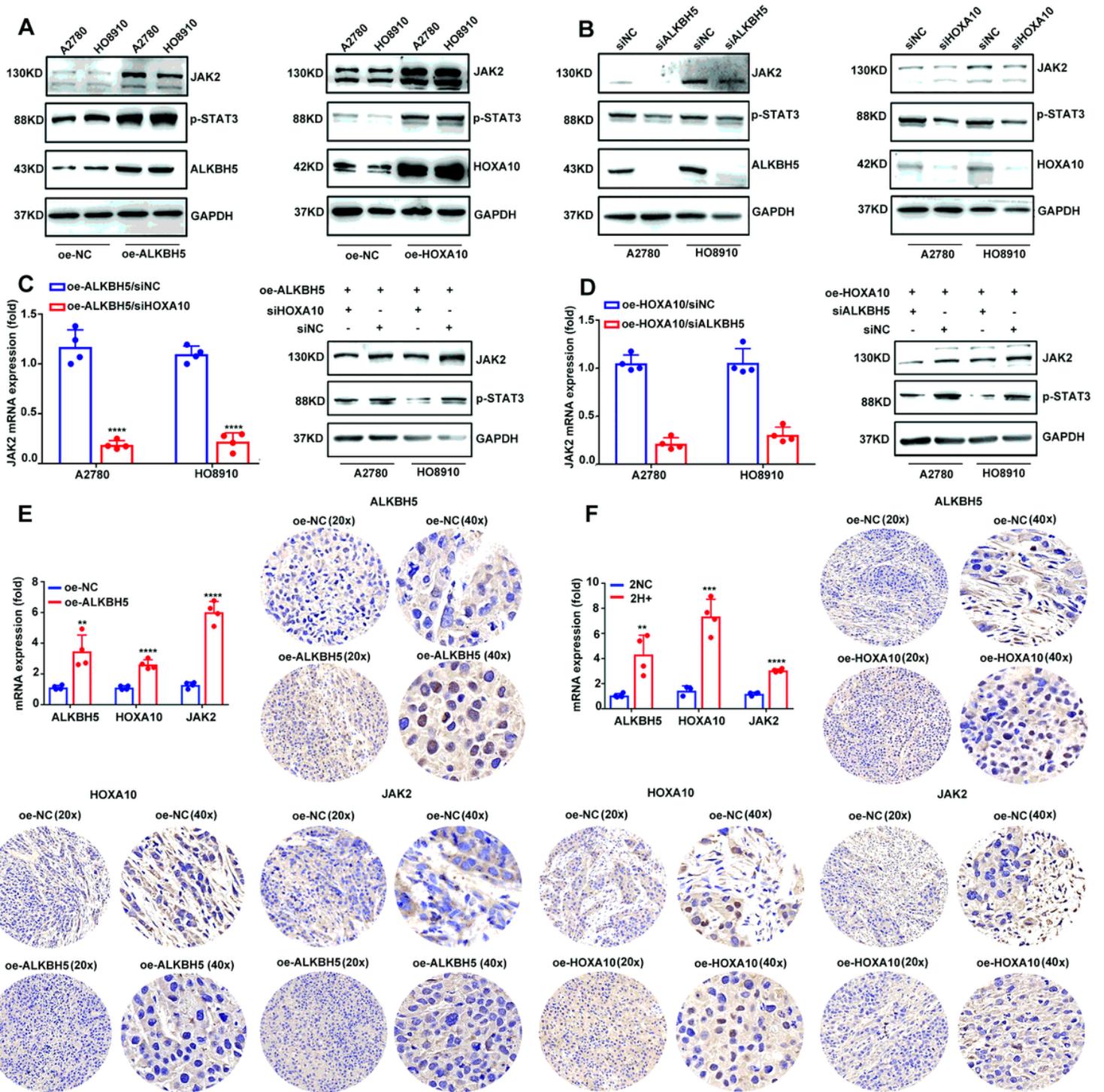


Figure 6

ALKBH5-HOXA10 loop overexpression activates the JAK2/STAT3 signaling pathway (A and B) Up- and downregulation of the ALKBH5-HOXA10 loop promotes and inhibits JAK2 expression, respectively, and the phosphorylation level of STAT3. (C and D) Activation of the JAK2/STAT3 pathway can be rescued by knocking down ALKBH5 or HOXA10. (E and F) The IHC assays show ALKBH/HOXA10/JAK2 regulation in the xenograft model.

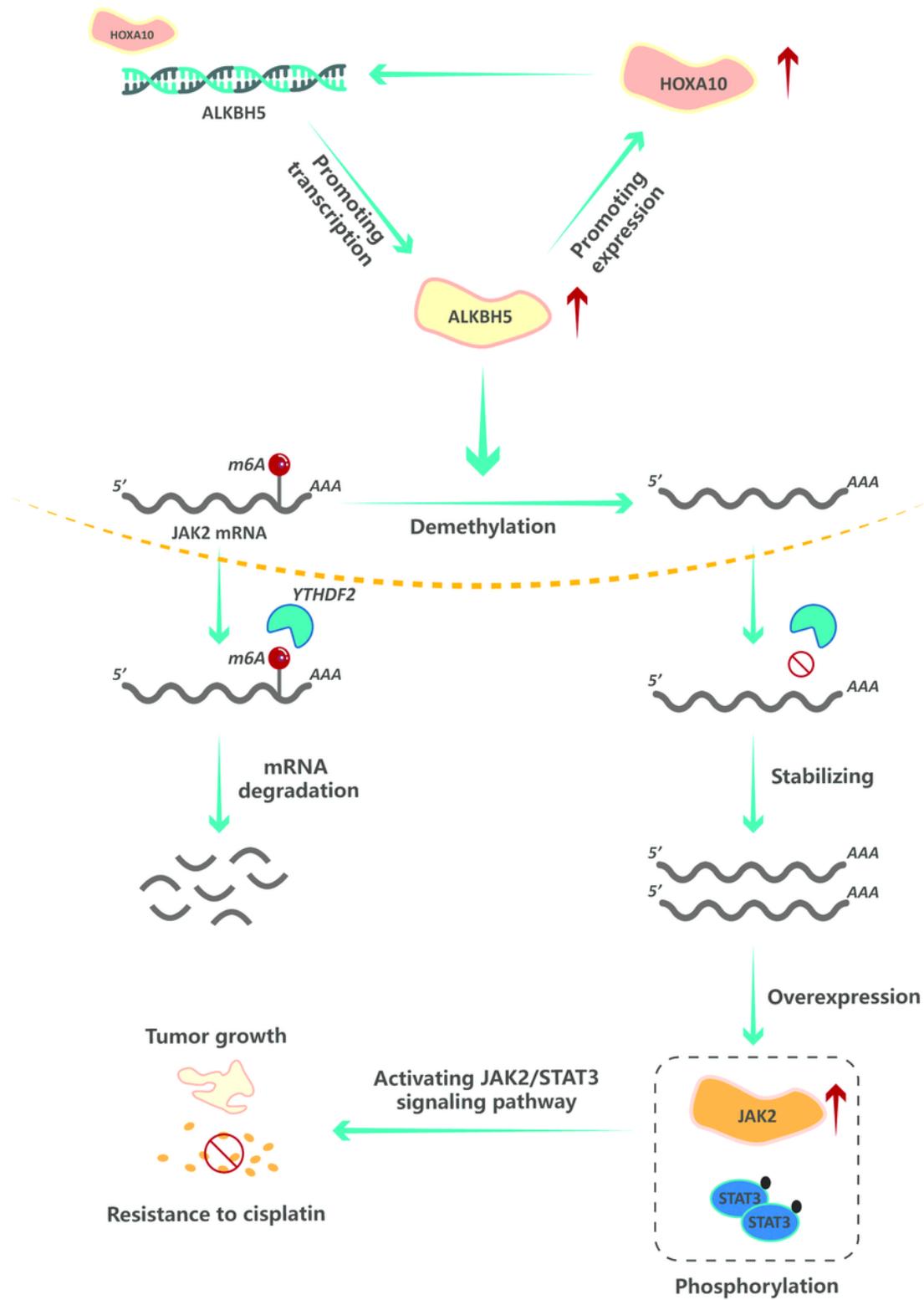


Figure 7

Graphic abstract Overview of the present study

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